DECLARATION

I, Katsuhiro YOSHIDA of 238, Nishikananoi, Showa-machi, Kitakatsushika-gun, Saitama-ken, Japan do hereby declare that I am well acquainted with the Japanese language and English language and the attached English translation of an officially certified copy of Japanese Patent Application No.2000-193133 is a true and correct translation to the best of my knowledge and belief from the Japanese language into English language.

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K. Yoshida

Katsuhiro YOSHIDA

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[Title of Invention] A novel quantitative, polymorphous analysis method.

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[Inventor]

[Address or Domicile] c/o National Institute of Bioscience and Human-Technology, 1-3, Higashi 1 Chome, Tukuba-shi, Ibaraki, Japan

[Name] Ryuichiro KURANE

[Inventor]

[Address or Domicile] c/o National Institute of Bioscience and Human-Technology, 1-3, Higashi 1 Chome, Tukuba-shi, Ibaraki, Japan

[Name] Takahiro KANAGAWA

[Inventor]

[Address or Domicile] c/o National Institute of Bioscience and Human-Technology, 1-3, Higashi 1 Chome, Tukuba-shi, Ibaraki, Japan

[Name] Yoichi KAMAGATA

[Inventor]

[Address or Domicile] c/o KANKYO ENGINEERING Co., Ltd.,, 1-9-8, Higashi-Kanda, Chiyoda-ku, Tokyo, Japan

[Name] Shinya KURATA

[Inventor]

[Address or Domicile] c/o KANKYO ENGINEERING Co., Ltd., 1-9-8, Higashi-Kanda, Chiyoda-ku, Tokyo, Japan

[Name] Kazutaka YAMADA

[Inventor]

[Address or Domicile] c/o KANKYO ENGINEERING Co., Ltd., 1-9-8, Higashi-Kanda, Chiyoda-ku, Tokyo, Japan

[Name] Toyokazu YOKOMAKU

[Applicant for Patent]

[Identification Number] 597031070

[Name] Japan Bioindustry Association

[Applicant for Patent]

[Identification Number] 000001144

[Name] Secretary of Agency of Industrial Science and Technology; Koji KAJIMURA.

[Applicant for Patent]

[Identification Number] 000156581

[Name] KANKYO ENGINEERING Co., Ltd.,

[Designated Representative]

[Identification Number] 220000404

[Name] Shinichi OHASHI, Director in National Institute of Bioscience and Human-Technology,

[Specified Matters for Representation] : Representative Designated by Patent applicant: Secretary of Agency of Industrial Science and Technology

[Agent]

[Identification Number] 100077698

[Patent Attorney]

[Name] Katsuhiro YOSHIDA

[Specified Matters for Representation] Agent Designated by

Patent applicant: KANKYO ENGINEERING Co., Ltd. and Japan Bioindustry Association

[Sub Agent]

[Identification Number] 100077698

[Patent Attorney]

[Name] Katsuhiro YOSHIDA

[Specified Matters for Representation] Agent Designated by Patent applicant: Secretary of Agency of Industrial Science and Technology

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[List of Material Submitted]

[Title of Material]	Specification	1
[Title of Material]	Drawings	1
[Title of Material]	Abstract	1
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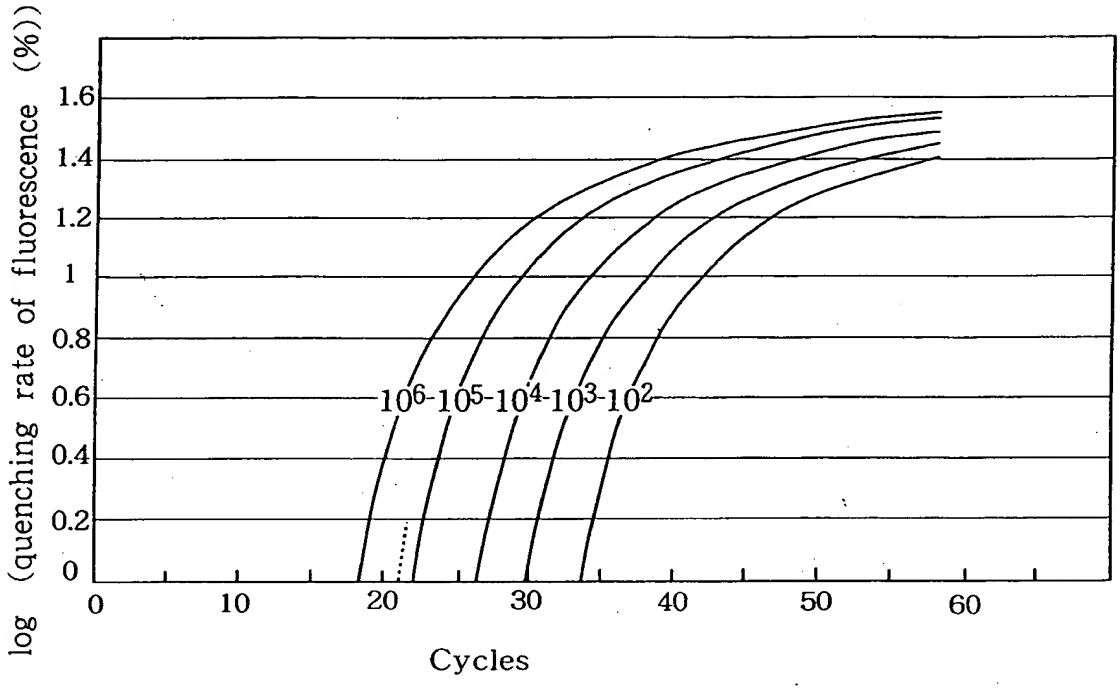
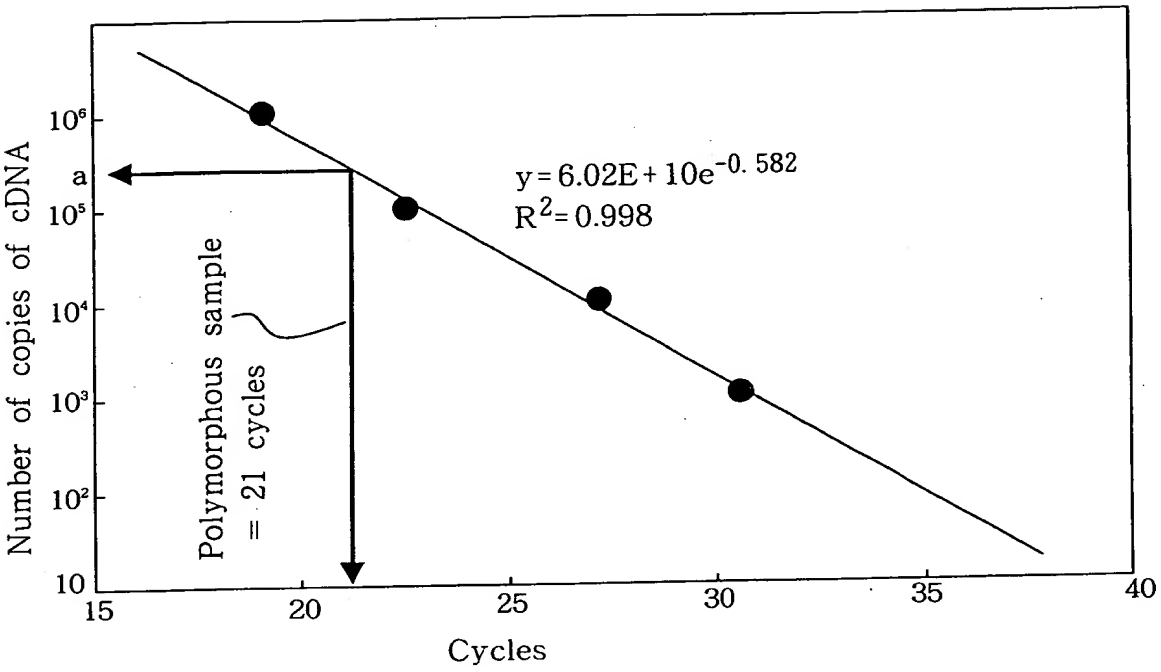
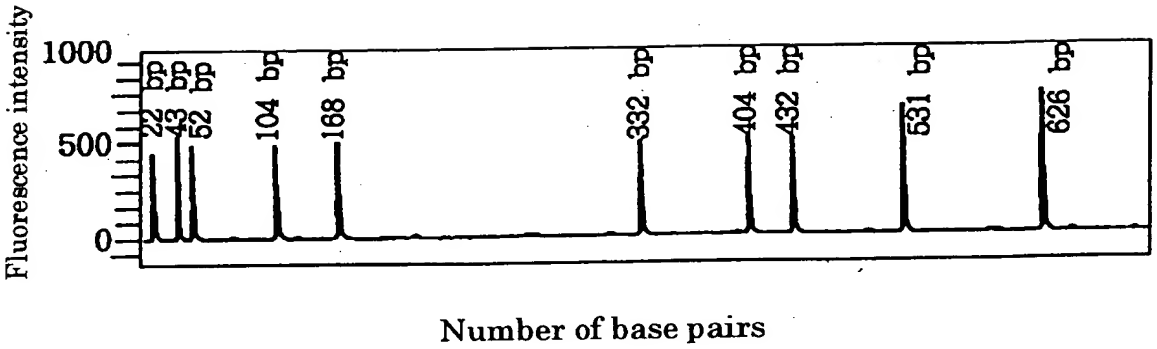
[Fig. 1]



[Fig. 2]


Number of copies of cDNA
10^6
a
10^5
10^4
10^3
10^2
10
Polymorphous sample
= 21 cycles
y = 6.02E + 10e$^{-0.582}$
R^2 = 0.998
15
20
25
30
35
40
Cycles

[Fig. 3]


Fluorescence intensity
1000
500
0
22 bp
43 bp
52 bp
104 bp
168 bp
332 bp
404 bp
432 bp
631 bp
626 bp
Number of base pairs

[Name of Document] DESCRIPTION

[Title of Invention] A novel quantitative, polymorphous analysis method

[Claims]

[Claim 1] A novel quantitative, polymorphous analysis method comprising amplifying a target gene by a quantitative gene amplification method, and performing a polymorphous analysis with respect to said target gene to determine an amount of said target gene and a polymorphous composition or amounts of individual components of said target gene.

[Claim 2] A quantitative, polymorphous analysis method according to claim 1, wherein said polymorphous analysis is T-RELP (terminal restriction fragment length polymorphism), RFLP (restriction fragment length polymorphism), SSCP (single strand conformation) or CFLP (cleavase fragment length polymorphism).

[Claim 3] A polymorphous analysis method according to claim 1 or 2, wherein said quantitative gene amplification method is a quantitative PCR method.

[Claim 4] A quantitative, polymorphous analysis method according to any one of claims 1 to 3, wherein said quantitative PCR is conducted using a fluorescence-quenching nucleic acid probe .

[Claim 5] A quantitative, polymorphous analysis method according to any one of claims 1 to 4, wherein said fluorescence-quenching probe is labeled at an end portion thereof with said fluorescent dye, and has a base sequence designed such that, when said probe hybridizes at said end portion thereof to said target nucleic acid, at least one G (guanine) base exists in a base

sequence of said target nucleic acid at a position 1 to 3 bases apart from an end base of said target nucleic acid hybridized with said probe, whereby said fluorescent dye is reduced in its fluorescent intesity when said nucleic acid probe labeled with said fluorescent dye hybridizes to said target nucleic acid.

[Claim 6] A quantitative, polymorphous analysis method according to any one of claims 1 to 5, wherein said said fluorescence-quenching probe is labeled at a 3' end thereof with said fluorescent dye.

[Claim 7] A quantitative, polymorphous analysis method according to claims 1 to 5, wherein said fluorescence-quenching probe is labeled at a 5' end thereof with said fluorescent dye.

[Claim 8] A quantitative, polymorphous analysis method according to claims 1 to 5, wherein said fluorescence-quenching probe is labeled at an end portion thereof with said fluorescent dye, and said nucleic acid probe has a base sequence designed such that, when said probe hybridizes to said target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at said end portion, whereby said fluorescent dye is reduced in fluorescence emission when said nucleic acid probe labeled with said fluorescent dye hybridizes to said target nucleic acid.

[Claim 9] A quantitative, polymorphous analysis method according to claim 8, wherein said fluorescence-quenching probe is labeled at said 3'end thereof with said fluorescent dye.

[Claim 10] A quantitative, polymorphous analysis method according to claim 8, wherein said fluorescence-quenching probe is labeled at said 5'end

thereof with said fluorescent dye.

[Claim 11] A quantitative, polymorphous analysis method according to any one of claims 1 to 4, wherein said fluorescence-quenching probe is labeled at a phosphate group.

[Claim 12] A quantitative, polymorphous analysis method according to claims 4, 10 or 11, wherein said quantitative, polymorphous analysis method comprises using said fluorescence-quenching probe as a primer in the quantitative PCR method, and measuring the amount of decrease in the fluorescence-emission for the dye.

[Claim 13] A quantitative, polymorphous analysis method according to any one of claims 4 to 12, wherein said quantitative PCR is a real-time monitoring quantitative PCR method.

[Claim 14] A method for analyzing data obtained by the real-time monitoring quantitative PCR method, comprising a processing step for correcting an intensity value of fluorescence in a reaction system, said intensity value being available after said target nucleic acid has hybridized to said nucleic acid probe labeled with said fluorescent dye, in accordance with an intensity value of fluorescence in said reaction system available after a probe-nucleic acid hybrid complex so formed has been denatured.

[Claim 15] A quantitative, polymorphous analysis method according to any one of claims 4 to 13, wherein said quantitative, polymorphous analysis method has a processing step for correcting data obtained by the real time-monitoring quantitative PCR method according to Claim 14.

[Claim 16] A reagent kit for use in the quantitative PCR method according to any one of claims 1 to 13, wherein said kit includes or is

accompanied by a fluorescence-quenching probe according to any one of claims 4-11.

[Claim 17] A reagent kit for analyzing or determining quantitatively the polymorphism of a target nucleic acid, wherein said kit includes or is accompanied by a reagent kid for use in the quantitative PCR method according to Claim 15.

[Claim 18] a computer-readable recording medium, wherein a program for making a computer perform the data analysis method according to Claim 14 has been already recorded.

[Claim 19] A data analyzer for a real time-monitoring quantitative PCR method, wherein said data analyzer is equipped with means for performing a data analyzing method according to Claim 14.

[Claim 20] A computer-readable recording medium for the analysis of data obtained by the quantitative polymorphous analysis method, wherein the said computer-readable recording medium recording is in a combination of a program for making a computer perform a method for analyzing data obtained by the quantitative polymorphous analysis method according any one of Claims 1 to 13 or Claim 15 with a program for making a computer perform a method for analyzing data according to Claim 14

[Claim 21] A data analyzer for use in the quantitative polymorphous analysis method, wherein said data analyzer is equipped with a data analyzer for the PCR method according to Claim 19.

[Detailed Explanation of Invention]

[0001].

[Technical Field]

The present invention is related to a novel quantitative, polymorphous analysis method, a gene amplification method used in the method, a novel method for analyzing data obtained by the quantitative gene amplification method, a reagent kit for the quantitative PCR method, a computer-readable recording medium for analyzing data obtained by a PCR method in which a program for making a computer perform a method for analyzing data obtained by the quantitative PCR method has already been recorded, a computer-readable recording medium for analyzing data obtained by a PCR method in which a program for making a computer perform procedures for analyzing data obtained by the quantitative polymorphous method has already been recorded, an analyzer for analyzing data obtained by a quantitative PCR method and an analyzer for analyzing data obtained by a quantitative polymorphous analysis method.

[0002]

[Prior Art]

Substantial technical improvements have been made on methods for amplifying a target gene by PCR [Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids, Enzymes), 35(17), KYORITSU SHUPPAN CO., LTD. (1990)] and conducting a polymorphous analysis on the target gene so amplified, and these polymorphous analysis methods have now found wide-spread utility in various fields such as medical field [Jikken Igaku (Laboratory Medicine), 15(7), Yodosha (1997)]. Various diseases, especially immune-related diseases have hence been elucidated from genes, thereby obtaining certain successful outcomes.

[0003]

Conventional polymorphous analyses are, however, each conducted using PCR methods which do not have quantitativeness; the methods do not have so a specificity as to amplify only one gene among genes having similar base sequences (for example, only a gene sequence of the 16SrRNA gene with respect of a specified microbe among all DNAs extracted from active sludge); it has been unable to make an analysis to such an extent as the amount or polymorphous composition of the initial gene before the amplification of the target gene.

[0004]

[Problem to be Solved]

With the foregoing in view, the present invention also has as an object thereof the provision of a novel polymorphous analysis method for easily and quickly determining an initial concentration of a target genes and a polymorphous composition of a target gene before the amplification of the gene; a novel quantitative PCR method usable for the polymorphous analysis method, a fluorescence-quenching probe usable for the quantitative PCR method, a novel method for analyzing data obtained by the quantitative PCR, a reagent kit usable for the quantitative PCR method, a reagent kit usable for the quantitative polymorphous analysis method, a computer-readable recording medium with programmed procedures, which are required to make a computer perform a method for analyzing data obtained by the quantitative PCR method, a computer-readable recording medium with programmed procedures, which are required to make a computer perform a method for analyzing data obtained by the quantitative

polymorphous analysis method, an analysis system for the quantitative PCR method, and an analysis system for the quantitative polymorphous analysis.

[0005]

[Means for Solving Problem]

To achieve the above-described objects, the present inventors have proceeded with a variety of investigations and have obtained findings such that, subsequent to the amplification of a target gene using a quantitative gene amplification method, they had performed a polymorphous analysis to be able to determine a novel polymorphous analysis method for easily and quickly determining an initial concentration of a target genes and a polymorphous composition of a target gene before the amplification of the gene. The present invention has been completed based on the above findings.

[0006]

That is, the present invention provides: a method for amplifying quantitatively a gene, specifically, a novel method for performing a quantitative polymorphous analysis comprising amplifying a target gene by a quantitative PCR method, performing a polymorphous analysis in respect to the amplified gene, determining the amount of the target gene before the amplification, identifying polymorphisms, determining a ratio of the existing amount thereof, determining quantitatively the amount of an individual polymorphism and analyzing quantitatively on the obtained data; a novel quantitative PCR method used for the quantitative polymorphous analysis; a fluorescence-quenching probe used for the quantitative PCR method; a novel analysis for analyzing the data obtained by the quantitative

PCR method; a reagent kit used for the quantitative PCR method, a reagent kit used for the quantitative polymorphous analysis method, a computer-readable recording medium with programmed procedures, which are required to make a computer perform a method for analyzing data obtained by the quantitative PCR method; a computer-readable recording medium with programmed procedures, which are required to make a computer perform a method for analyzing data obtained by the quantitative polymorphous analysis method, an analysis system for the quantitative PCR method, and an analysis system for the quantitative polymorphous analysis.

[0007]

[Preferable Embodiment of Invention]

The present invention will next be described in further detail based on certain preferred embodiments.

At first, the technical terms employed in the present invention will be defined as described below.

The term "target gene" means a nucleic acid or a gene which is intended to be assayed/determined or detected quantitatively. The examples of the gene include DNAs, RNAs, PNAs, oligodeoxyribonucleotides, and oligoriboxynucleotides. Further the gene may also be mixed with other various genes, and additionally with proteins, such higher molecular compounds as polysaccharides and the like, and such lower molecular compounds as amino acids, sugars, vitamins, inorganic compounds and the like. Further still, the gene may exist in a cell. Namely, particular limitation is not imposed on the gene. The cell may be a eukaryotic or prokaryotic cell. Further more, the cell may be mixed with other various cells. The gene may

be in a purified form or in a non-purified form and further may be lower or higher in its concentration. For example, it is a specified gene to be analyzed in a complex microbe system or a symbiotic microbe system. On the need of purification of the gene, the purification may be conducted in a conventional method. In addition, a purification kit may be used which is available in a market.

[0008]

"PCR" means a polymerase chain reaction, and is at present a reaction used generally in the molecular biology, molecular cytology, gene engineering and the like.

A "quantitative PCR" means a method for determining or detecting an existing amount of a target gene before the PCR.

A "fluorescence quenching probe" means a probe designed so that, on hybridization of a probe labeled with a fluorescent dye with a target gene, a fluorescent intensity for the dye is caused.

[0009]

A "primer in PCR", a "nucleic acid probe" and terms "hybridize" and "hybridization" have the same meanings as the corresponding terms employed at present in the molecular biology and genetic engineering.

[0010]

The term “polymorphous” or “polymorphism” as used herein means biological polymorphous or polymorphism. In the present invention, it means especially the polymorphism of a gene (RNA, DNA, gene) on which the polymorphism is brought about. It has the same meaning as commonly employed these days in molecular biology.

The term "polymorphous analysis" means to analyze and/or determine what polymorphism a gene has.

[0011]

Currently-available examples of the polymorphous method include SSOP (sequence specific oligonucleotide probe) method, RELP (restriction fragment length polymorphism) method, T-RFLP (terminal restriction fragment length polymorphism) method, SSCP (single strand conformation) method, MPH method, CFLP (cleavase fragment length polymorphism) method, SSP (ssequence specific primer) method, PHFA (preferential homoduplex formation formation assay) method, SBT (sequence base typing) method [PCR Ho, Riyo no Tebiki(PCR Methods, Manual for Their Use), Chugai Medical Publishing Co., Ltd. (1998); Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids, Enzymes), 35(17), KYORITSU SHUPPAN CO., LTD. (1990); Jikken Igaku (Laboratory Medicine), 15(7)(special number), Yodosha (1997)]. T-RELP method or CFLP method can be especially suitably applied, although the methods currently used in polymorphous analyses are all usable in the present invention.

[0012]

Features of the polymorphous analysis method will hereinafter be described specifically in order.

The first feature resides in a quantitative gene amplification method. Any quantitative gene amplification method can be adopted insofar as it has quantitativeness. For example, PCR methods can be adopted suitably. Among these, quantitative PCR methods and real-time monitoring, quantitative PCR methods are more preferred.

Examples of conventionally-known, quantitative PCR methods can include RT-PCR, RNA-primed PCR, Stretch PCR, reversed PCR, PCT making use of an Alu sequence, multiple PCR, PCR making use of a mixed primer, and PCR making use of PNA.

[0013]

According to these conventionally-known, quantitative PCR methods, a target gene is amplified by cycling the temperature between a low temperature and a high temperature in the presence of Mg ions while using dATP, dGTP, dCTP and dTTP or dUTP, a target gene (DNA or RNA), Taq polymerase, a primer and a nucleic acid probe labeled with a fluorescent dye or an intercalator, and an increase in the emission of fluorescence from the fluorescent dye in the course of the amplification is monitored in a real-time manner [Jikken Igaku (Laboratory Medicine), 15(7), 46-51, Yodosha (1997)].

[0014]

The quantitative PCR method, which makes use of the fluorescence -quenching probe according to the present invention, is a method in which the probe labeled with the fluorescent dye is used; the method is a quantitative PCR method that makes use of a probe designed such that, on the hybridization of the probe with a target gene, the fluorescence-emission for the labeled dye is decreased or quenched.

For example, a fluorescence quenching probe is labeled at an end thereof with a fluorescent dye, and its base sequence is designed such that, when the probe hybridizes at the end portion thereof to a target gene, at least one G (guanine) exists in a base sequence of the target gene at a position 1 to 3 bases apart from the portion of an end base pair of the target

gene hybridized with the probe, whereby the fluorescent dye is reduced in fluorescence emission when the probe hybridizes to the target gene. Among these, fluorescence quenching probes each of which is labeled at a 3'end or 5'end thereof with a fluorescent dye are more preferred.

[0015]

Preferably, the fluorescence quenching probe is labeled at the end thereof with the fluorescent dye, and its base sequence is designed such that, when the probe hybridizes to the target gene, base pairs of the hybrid complex of the probe and the target gene forms at least one G (guanine) and C (cytosine) pair (GC base pair) at the end thereof, whereby the fluorescent dye is reduced in the intensity of fluorescence when the probe hybridizes to the target gene.

[0016]

If the 5' or 3' end cannot be designed to G or C due to the base sequence of a target gene, the objects of the present invention can also be adequately achieved by adding 5'-guanylic acid or 5'-cytidylic acid to the 5'end of an oligonucleotide designed as a primer from the base sequence of the target nucleic acid. The expression "fluorescence quenching probe" as used herein is, therefore, defined to embrace not only probes designed based on the base sequence of the target nucleic acid but also probes added at the 3' or 5'ends thereof, preferably the 5'ends thereof with 5'-guanylic acid or 5'-cytidylic acid.

[0017]

The term "fluorescent dye" as used herein means fluorescent dyes or the like, which are generally used for the determination or detection of

nucleic acids by labeling nucleic acid probes. Illustrative of such fluorescent dyes are fluorescein and derivatives thereof [for example, fluorescein isothiocyanate (FITC) and its derivatives]; Alexa 488, Alexa 532, cy3, cy5, 6-joe, EDANS; rhodamine 6G (R6G) and its derivatives [for example, tetramethylrhodamine (TMR), tetramethylrhodamine isothiocyanate (TMRITC), x-rhodamine, Texas red, "BODIPY FL" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY FL/C3" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY FL/C6" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY 5-FAM" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY TMR" (trade name, product of Molecular Probes, Inc., U.S.A.), and derivatives thereof (for example, "BODIPY TR" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY R6G" (trade name, product of Molecular Probes, Inc., U.S.A.), "BODIPY 564" (trade name, product of Molecular Probes, Inc., U.S.A.), and "BODIPY 581" (trade name, product of Molecular Probes, Inc., U.S.A.)). Among these, FITC, EDANS, Texas red, 6-joe, TMR, Alexa 488, Alexa 532, "BODIPY FL/C3" and "BODIPY FL/C6" are preferred, with FITC, TMR, 6-joe, "BODIPY FL/C3" and "BODIPY FL/C6" being more preferred.

[0018]

The probe labeled with a fluorescent dye, making use in the present invention, comprises a oligodeoxyribonucleotide or a oligoribonucleotide; the oligonucleotide contains 5 to 50 bases, preferably 10 to 25 bases, especially preferably 15 to 20 bases. No particular limitation is imposed on its base sequence insofar as the probe hybridizes specifically to the target

gene.

[0019]

The oligonucleotide in the fluorescence-quenching probe labeled with a fluorescent dye according to the present invention can be produced by a conventional production process for general oligonucleotides. It can be produced, for example, by a chemical synthesis process or by a microbial process which makes use of a plasmid vector, a phage vector or the like (Tetrahedron Letters, 22, 1859-1862, 1981; Nucleic Acids Research, 14, 6227-6245, 1986). Further, it is suitable to use a nucleic acid synthesizer currently available on the market (for example, "ABI 394", manufactured by Perkin-Elmer Corp., U.S.A.).

In the case when the fluorescence-quenching probe is used as a primer for a quantitative PCR method, the probe may be designed in any type of a forward type, a reverse type or a bachward type in the preparation thereof.

[0020]

To label the oligonucleotide with the fluorescent dye, desired one of conventionally-known labeling methods can be used (Nature Biotechnology, 14, 303-308, 1996; Applied and Environmental Microbiology, 63, 1143-1147, 1997; Nucleic Acids Research, 24, 4532-4535, 1996). To conjugate a fluorescent dye to the 5'end, the 5'-end is made to dephosphorylate; a linker or spacer, for example, $-(CH_2)_n-SH$ or $-(CH_2)_n-NH_2$ is first introduced into a OH group at the 5'end by a method known *per se* in the art. Further, the spacer or linker also may be at the phosphate group of the ribose. A dye may be conjugated to the spacer or linker thereof.

[0021]

As such a linker- or spacer-introduced derivative is available on the market, a commercial product may be purchased (Midland Certified Reagent Company). In the above-mentioned example, n ranges from 3 to 12, with 6 to 10 being preferred. The oligonucleotide can be labeled by reacting an fluorescent dye to a SH- or NH_2- group of the linker or spacer. Reversed phase chromatography or the like to provide a nucleic acid probe for use in the present invention can purify the thus-synthesized oligonucleotide, which is labeled with the fluorescent dye.

[0022]

According to the quantitative PCR method making use of the fluorescence quenching probe, the target gene can be easily and specifically amplified in short time. When a fluorescence quenching probe labeled at the 5'end thereof with a fluorescent dye is used, a target gene labeled at the 5'end thereof with the fluorescent dye is amplified [Jikken Igaku (Laboratory Medicine), 15(7), Yodosha (1997)].

[0023]

As a thermal cycler for use in the quantitative PCR method, any one of various equipment currently available on the market can be conveniently used no matter whether or not it permits real-time monitoring. Particularly preferred examples of equipment, which permit real-time monitoring, can include "ABI PRISM™ 7700 Sequence Detection System" (SDS 7700)(Perkin-Elmer Applied Biosystem, Inc., U.S.A.) and "LightCycler"™ System" (Roche Diagnostic GmbH, Germany).

[0024]

Amplification of a gene can be attained under amplifying reaction

conditions known to date. It is generally desired to proceed with amplification to an amplification degree which is commonly used. In the course of the amplification of the target gene, the intensity of fluorescence is measured by a fluorimeter. Changes in the intensity of fluorescence are proportional with amplified amounts of the gene. Plotting of the changes in the intensity of fluorescence as a function of time (cycles in the case of PCR) on an ordinary graph paper gives an S-shaped (sigmoid) curve, whereas their plotting on a semilog graph paper gives a line, which linearly increases in the beginning like an exponential function but then forms a curve which reaches a gentle plateau.

As the degree of amplification of the target gene, in other words, the time to stop the amplifying reaction of the gene to improve the quantitativeness of the initial amount of the gene before starting PCR depends upon the purpose of the polymorphous analysis, no particular limitation is imposed thereon. Described specifically, when a polymorphous system is analyzed for only priority polymorphism, it is suited to amplify the target gene for a desired time from the initial observation of a change in the intensity of fluorescence until before the above-described plateau is reached. It is most preferable to stop the reaction in an exponential growth phase [i.e., before reaching a midpoint of the sigmoid curve (a point where a derivative of the curve becomes 0)]. When it is desired to analyze all polymorphous species contained in the polymorphous system, it is desired to conduct several experiments in a trial and error manner to determine a degree of amplification considered to be the best and then to amplify the gene to such extent that genes, which show polymorphism in the reaction system, can all

be observed. A method - in which amplification is conducted by dividing it in plural stages, in other words, an experiment is conducted at plural degrees of amplification and the results are analyzed as a whole - can also be adopted appropriately, because minor polymorphous species tend to draw a sigmoid curve having large time lags.

[0025]

When the quantitative PCR method, especially the real-time monitoring quantitative PCR method is performed using the fluorescence quenching probe of this invention as a primer, the fluorescence quenching probe as the primer is used repeatedly for the amplification of the target gene so that the target gene labeled at the 5'end thereof with the fluorescent dye is amplified. The amplified target gene then hybridizes to the corresponding target gene. When this hybridization takes place, the intensity of fluorescence decreases. It is therefore only required to conduct the amplifying reaction to the best degree of amplification in a similar manner as described above while tracing decreases in the intensity of fluorescence. This quantitative PCR method can also be conducted under similar reaction conditions as the conventional PCR methods. Accordingly, amplification of a target gene can be conducted in a reaction system the Mg ion concentration of which is low (1 to 2 mM) or, as was known conventionally, is high (2 to 4 mM).

[0026]

It is preferred to prepare a working line for the target gene by using a target gene before the amplifying reaction of the target gene. A description will now be made about an illustrative case in which the above-described

fluorescence quenching probe was used as a primer and the real-time monitoring quantitative PCR method was conducted.

Plotting of decreases in the intensity of fluorescence as a function of cycles on an ordinary graph paper gives an S-shaped (sigmoid) curve. An exponential relation exists between the number of cycles at a time point where the rate of decrease was the greatest and the initial number of copies of the target gene (the number of copies before the initiation of PCR), that is, the target gene in the initial stage. Advanced preparation of a target straight line, which represents the correlation between the number of cycles and the number of copies at that time point, makes it possible to determine the initial number of copies of the target gene in an unknown sample, namely, the initial amount of the target gene.

Incidentally, the above-described quantitative PCR method making use of the fluorescence quenching probe is a novel method developed by the present inventors.

[0027]

As the second feature of the quantitative polymorphous analysis method, it is an analysis method for analyzing data obtained by the quantitative PCR method. As a matter of fact, it is nothing but a method for analyzing data obtained by the above-described quantitative PCR method. This analysis method is currently most suited for determining the initial amount of the target gene as accurately as possible.

[0028]

At first, the present invention relates to a data analysis system

The present invention has three features.

A first feature resides in a processing step for correcting a fluorescence intensity of a reaction system, which is measured when the nucleic acid amplified in each cycle is conjugated with the fluorescent dye or when the amplified nucleic acid hybridizes to a nucleic acid probe according to the present invention in the method for analyzing data obtained by the real-time quantitative PCR method, by a fluorescence intensity of the reaction system as obtained when the above-described conjugate of the fluorescent dye and the nucleic acid or the fluorescent dye-nucleic acid conjugate or the above-described hybrid complex of the nucleic acid probe of the present invention and the target nucleic acid or the nucleic acid hybrid complex has dissociated in each cycle, namely, the first feature resides in a correction-processing step.

[0029]

Any correction processing can be used as the correction processing in the correction processing step insofar as it conforms with the objects of the present invention. Specifically, correction processing including a processing step by the following formula (1) or formula (2) can be exemplified.

$$f_n = f_{hyb,n}/f_{den,n} \qquad (1)$$

$$f_n = f_{den,n}/f_{hyb,n} \qquad (2)$$

where

f_n: correction-processed value in an n^{th} cycle as calculated in accordance with the formula (1) or formula (2),

$f_{hyb,n}$: intensity value of fluorescence of the reaction system available after the amplified nucleic acid has conjugated to the fluorescent

dye or the amplified nucleic acid has hybridized to the nucleic acid probe labeled with the fluorescent dye in the n^{th} cycle, and

$f_{den,n}$: intensity value of fluorescence of the reaction system available after the fluorescent dye-nucleic acid conjugate or the nucleic acid hybrid complex has dissociated in the n^{th} cycle.

This step includes a sub-step in which correction-processed values obtained by the above-described processing are displayed on a computer display and/or the correction-processed values are likewise displayed and/or printed in the form of a graph as a function of cycles.

[0030]

A second feature resides in a data analysis method, which comprises introducing correction-processed values, which have been calculated in accordance with the formula (1) or formula (2) in individual cycles, into the following formula (3) or formula (4) to calculate rates or percentages of changes in fluorescence between samples in the individual cycles:

[0031]

$$F_n = f_n/f_a \qquad (3)$$

$$F_n = f_a/f_n \qquad (4)$$

where

F_n: rate or percentage of a change in fluorescence in an n^{th} cycle as calculated in accordance with the formula (3) or formula (4),

f_n: correction-processed value calculated in the n^{th} cycle as calculated in accordance with the formula (1) or formula (2), and

f_a: correction-processed value calculated in a given cycle before a change in f_n is observed as calculated in accordance with the formula (1) or

formula (2), and in general, a correction-processed value, for example, in one of 10^{th} to 40^{th} cycles, preferably one of 15^{th} to 30^{th} cycles, more preferably one of 20^{th} to 30^{th} cycles is adopted; and comparing the rates or percentages of changes in fluorescence.

This step includes a sub-step in which calculated values obtained by the above-described processing are displayed on a computer display and/or are printed or comparative values or the calculated values are likewise displayed and/or printed in the form of a graph as a function of cycles. This sub-step may be applied or may not be applied to the correction-processed values obtained by the formula (1) or formula (2).

[0032]

A third feature resides in a data analysis method, which comprises the following processing steps: 3.1) performing processing in accordance with the following formula (5), (6) or (7) by using data of rates or percentages of changes in fluorescence as calculated in accordance with said formula (3) or (4):

$$\log_b(F_n),\ \ln(F_n) \quad (5)$$

$$\log_b\{(1-F_n) \times A\},\ \ln\{(1-F_n) \times A\} \quad (6)$$

$$\log_b\{(F_n-1) \times A\},\ \ln\{(F_n-1) \times A\} \quad (7)$$

[0033]

where

A,b: desired numerical values, preferably integers, more preferably natural numbers and, when A=100, b=10, $\{(F_n-1) \times A\}$ is expressed in terms of percentage (%), and

F_n: rate or percentage of a change in fluorescence in an n^{th} cycle as

calculated in accordance with the formula (3) or formula (4),

[0034]

3.2) determining a cycle in which said processed value of said processing step 3.1) has reached a constant value,

3.3) calculating a relational expression between cycle of a nucleic acid sample of a known concentration and the number of copies of said target nucleic acid at the time of initiation of a reaction, and

3.4) determining the number of copies of said target nucleic acid in an unknown sample upon initiation of PCR.

Preferably, these steps are performed in the order of 3.1) → 3.2) → 3.3) → 3.4).

[0035]

Each of these steps 3.1) to 3.3) may include a sub-step in which processed values obtained by the corresponding processing are displayed on a computer display and/or the processed values are likewise displayed and/or printed in the form of a graph as a function of cycles. The step 3.4) should include at least a printing sub-step as the processed values obtained in the step 3.4) have to be printed, although the processed values obtained in the step 3.4) may also displayed on a computer display.

Incidentally, the correction-processed values obtained by the formula (1) or (2) and the calculated values obtained by the formula (3) or (4) may be or may not be displayed on a computer display and/or printed in the form of graphs as a function of cycles, respectively. These displaying and/or printing sub-steps may, therefore, be added as needed.

[0036]

Further, the present invention is related to a reagent kit for use for the above quantitative PCR method, and a computer-readable recording medium wherein the program for making a computer perform the data analysis method as described above is recorded.

Further still, a data-analyzing system with means for performing the above data-analyzing method.

[0037]

The third feature of the present invention relates to a method for analyzing polymorphism with respect to genes amplified by the quantitative PCR method according to the present invention.

Now, this polymorphous analysis method will be described specifically. Among various polymorphous analysis methods, T-RFLP can be suitably used in the present invention. As an example of the present invention, a gene is amplified by a quantitative PCR method making use of a fluorescence quenching probe as a primer, especially by a real-time monitoring quantitative PCR method, and the initial amount of the gene before PCR is determined. Further, a detailed description will be made about a method for analyzing polymorphism of the amplification products by T-RFLP. Incidentally, the gene amplified by using the fluorescence quenching probe as a primer is labeled at the 5'end thereof with the fluorescent dye useful in the practice of the present invention.

(1) Firstly, the amplification products are digested by a restriction endonuclease. As this restriction endonuclease, the currently known restriction endonucleases are all usable. Illustrative are Bso FI, Hha I, Hph I, Mnl I, Rca I, Alu I and Msp I. Among these, preferred are Hha I,

Alu I and Msp I, with Hha I being most preferred. As digesting reaction conditions, conditions generally employed for the currently known genes can be used. If Hha I is chosen as a restriction endonuclease, for example, it is reacted at 37℃ for 6 hours at a restriction endonuclease concentration of 10 units.

[0038]

(2) Gene fragments digested as described above can preferably be thermally modified into single-stranded forms. This modification treatment can also be conducted under usual conditions known to the public. For example, they are treated at 97℃ for 5 minutes and then chilled in ice.

[0039]

(3) Analysis and determination of gene fragments

In the polymorphous analysis method of the present invention, only the gene fragments labeled with the fluorescent dye are analyzed and determined by electrophoresis, HPLC, sequencer or the like.

Described specifically, individual bands and band peaks are detected based on fluorescence intensities. This detection can be conducted using an ordinary analyzer currently available on the market. Examples of the analyzer can include "ABI 373A" (a sequencer manufactured by Applera Corp-Applied Biosystems Group), "ABI 377" (a sequencer manufactured by Applera Corp-Applied Biosystems Group), and "Biofocus 3000" (manufactured by Bio-Rad Laboratories, Inc.).

[0040]

In the present invention, appearance of plural bands or plural peaks in the above-described analysis means existence of polymorphism. A single

band or a single peak means non-existence of polymorphism. A fluorescence intensity ratio of individual bands or peaks obviously means a polymorphous ratio. As the amount of a target gene before PCR is determined in the quantitative PCR method of the present invention, multiplication of the determined value by the above-described polymorphous ratio makes it possible to determine the initial amounts of the individual species of the polymorphous gene.

A method for obtaining data with respect to polymorphism as described above has been successfully provided for the first time owing to the use of the quantitative PCR method making use of the fluorescence quenching probe of the present invention.

Further, a convenient reagent kit for quantitative polymorphous analysis can also be provided by either including or attaching a reagent kit for the quantitative PCR method.

In addition, additional recording of a program, which is adapted to make a computer perform an analysis of data of the above-described real-time monitoring quantitative PCR, in a computer-readable recording medium - in which a program for making the computer perform the analysis method of data obtained by the above-described polymorphous analysis method has already been recorded – can provide a more convenient, computer-readable recording medium for the analysis of data obtained by the quantitative polymorphous analysis method.

Moreover, combined arrangement of a data analyzer for PCR with a polymorphous analyzer equipped with means for performing the quantitative polymorphous analysis method can provide a more convenient

polymorphous analyzer.

[0041]

[Examples]

The present invention will next be described more specifically based on the following Examples. No limitation is imposed on the present invention by the examples.

Example 1

The base selectivity of a fluorescence-quenching probe according to the present invention - causing the fluorescence quenching phenomenon - to a target nucleic acid, that is, the base specificity, was investigated. Ten kinds of target genes (deoxyribooligonucleotides)(30 mer); poly a to poly j, were prepared by a DNA synthesizer, "ABI 394" (Perkin-Elmer Corp., U.S.A.)

[0042]

Also prepared were the below-described fluorescence-quenching probes according to the present invention, which were labeled with "BODIPY FL" at the 5'ends of the deoxyribooligonucleotides corresponding to the above target genes, respectively.

The deoxyribooligonucleotides, in which a $-(CH_2)_6\text{-}NH_2$ group had already been bonded to the phosphate groups of the 5'ends of the deoxyribooligonucleotides, were purchased from Midland Certified Reagent Company, U.S.A. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" was also purchased, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the deoxyribooligonucleotide. The kit

was caused to act on the above-purchased deoxyribooligonucleotides, whereby the fluorescence-quenching probes · according to the present invention · labeled with "BODIPY FL" (probes a to d, f to h) were synthesized. An examination about how much the fluorescent intensity would be decreased (in other words, the degree of quenching) was made under the below-described conditions when the probes were caused to hybridize to their corresponding target genes, and the specificity of the invention probes was examined.

[0043]

Further, the above synthesized product was purified as described below.

The synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M Na_2CO_3 / $NaHCO_3$ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (product of Pharmacia AB), whereby unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby a nucleic acid probe was obtained with a yield of 50% as calculated relative to 2 mM of the starting oligonucleotide.

[0044]

The conditions of the reversed phase chromatography:

Eluting solvent A: 0.05 N TEAA 5% CH_3CN

Eluting solvent B (for gradient elution): 0.05 N TEAA 40% CH_3CN

Column: CAPCEL PAK C18, 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40℃

Detection: 254 nm

[0045]

Name	Target deoxyribooligonucleotide
poly a	5'ATATATATTTTTTTTGTTTTTTTTTTTTTT3'
poly b	5'ATATATATTTTTTTTTGTTTTTTTTTTTTT3'
poly c	5'ATATATATTTTTTTTTTGTTTTTTTTTTTT3'
poly d	5'ATATATATTTTTTTTTTTGTTTTTTTTTTT3'
poly e	5'ATATATATTTTTTTTTTTTGTTTTTTTTTT3'

[0046]

Name	Target deoxyribooligonucleotide
poly f	5'ATATATATTTTTTTTCTTTTTTTTTTTTTT3'
poly g	5'ATATATATTTTTTTTTCTTTTTTTTTTTTT3'
poly h	5'ATATATATTTTTTTTTTCTTTTTTTTTTTT3'
poly i	5'ATATATATTTTTTTTTTTCTTTTTTTTTTT3'
poly j	5'ATATATATTTTTTTTTTTTCTTTTTTTTTT3'

[0047]

Name	Invention probe
Probe a	3'TATATATAAAAAAAACAA5'-BODIPY FL/C6
Probe b	3'TATATATAAAAAAAAACA5'-BODIPY FL/C6
Probe c	3'TATATATAAAAAAAAAAC5'-BODIPY FL/C6
Probe d	3'TATATATAAAAAAAAAAA5'-BODIPY FL/C6

[0048]

Name	Invention probe
Probe f	3'TATATATAAAAAAAAGAA5'-BODIPY FL/C6
Probe g	3'TATATATAAAAAAAAAGA5'-BODIPY FL/C6
Probe h	3'TATATATAAAAAAAAAAG5'-BODIPY FL/C6

[0049]

(1) Components of hybridization mixture

Synthetic DNA	320 nM (final concentration)
Nucleic acid probe	80 nM (final concentration)
NaCl	50 mM (final concentration)
$MgCl_2$	1 mM (final concentration)
Tris-HCl buffer (pH 7.2)	100 mM (final concentration)
"MiliQ" purified water	1.6992 mL
Final whole volume	2.0000 mL

(2) Hybridization temperature: 51℃

(3) Measuring conditions

Exciting wave length : 543 nm

Measuring fluorescent color : 569 nm

[0050]

[Table 1]

Table 1

Nucleic acid probe	Target nucleic acid	Decrease in Fluorescence intensity (%)
a	a	-10
b	b	2
c	c	75
d	d	48
d	e	18
f	f	-8
g	g	-2
h	h	70
d	i	-6
d	j	-5

[0051]

The results are shown in Table 1. As is appreciated from Table 1, it is preferred to design the base sequence of a nucleic acid probe labeled with a

fluorescent dye such that, when the nucleic acid probe hybridizes to a target DNA (deoxyribooligonucleotide), at least one G (guanine) exists in the base sequence of the target DNA at a position 1 to 3 bases apart from an end base portion where the probe and the target DNA are hybridized with each other. From Table 2, it is also understood to be desired to design the base sequence of a nucleic acid probe labeled with a fluorescent dye such that, when the nucleic acid probe is hybridized with a target DNA, base pairs in the nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at the end portion.

[0052]

Example 2

Target nucleic acids and invention nucleic acid probes of the below-described base sequences were prepared. In a similar manner as in the preceding Example, an investigation was made about effects of the number of G(s) in each target nucleic acid and the number of G(s) in its corresponding invention nucleic acid probe.

[0053]

Name	Target deoxyribooligonucleotide
poly k	5'TATATATATATTTTTGGGGG3'
poly l	5'TATATATATATTTTTTGGGG3'
poly m	5'TATATATATTTTTTTTTGGG3'
poly n	5'TATATATATTTTTTTTTTGG3'
poly o	5'TATATATATTTTTTTTTTTG3'

[0054]

Name	Target deoxyribooligonucleotide

poly p 5'TATATATATATTTTTCCCCC3'

poly q 5'TATATATATATTTTTTCCCC3'

poly r 5'TATATATATTTTTTTTTCCC3'

poly s 5'TATATATATTTTTTTTTTCC3'

poly t 5'TATATATATTTTTTTTTTTC3'

poly u 5'TATATATATTTTTTTTTTTT3'

[0055]

Name	Invention probe
probe k	3'ATATATATATAAAAACCCCC5'-BODIPY FL/C6
probe l	3'ATATATATATAAAAAACCCC5'-BODIPY FL/C6
probe m	3'ATATATATATAAAAAAACCC5'-BODIPY FL/C6
probe n	3'ATATATATATAAAAAAAACC5'-BODIPY FL/C6
probe o	3'ATATATATATAAAAAAAAAC5'-BODIPY FL/C6

[0056]

Name	Invention probe
probe p	3'ATATATATATAAAAAGGGGG5'-BODIPY FL/C6
probe q	3'ATATATATATAAAAAAGGGG5'-BODIPY FL/C6
probe r	3'ATATATATATAAAAAAAGGG5'-BODIPY FL/C6
probe s	3'ATATATATATAAAAAAAAGG5'-BODIPY FL/C6
probe t	3'ATATATATATAAAAAAAAAG5'-BODIPY FL/C6
probe u	3'ATATATATATAAAAAAAAAA5'-BODIPY FL/C6

[0057]

[Table 2]

Table 2

Nucleic acid probe	Target nucleic acid	Decrease in Fluorescence intensity (%)
k	k	93
l	l	92
m	m	94
n	n	92
o	o	87
p	p	61
q	q	68
r	r	69
s	s	75
t	t	73
u	u	2

[0058]

As is appreciated from Table 2, the probe has preferably a base sequence designed such that, on hybridization of the fluorescence-quenching probe to a target nucleic acid, at least one base pair of G and C is formed in the hybridization complex.

[0059]

Example 3

Target nucleic acids and invention nucleic acid probes of the below-described base sequences were prepared in a similar manner as described above. The invention nucleic acid probes in this Example were each labeled at the 5'end portion of oligonucleotide with "BODIPY FL/C6". In a similar manner as in the preceding Example, an investigation was made about effects of the kind of bases in each target nucleic acid and the kind of bases in its corresponding invention nucleic acid probe.

[0060]

Name	Target deoxyribooligonucleotide
poly W	5'CCCCCCTTTTTTTTTTTT3'
poly X	5'GGGGGGAAAAAAAAAAAA3'
poly Y	5'TTTTTTCCCCCCCCCCCC3'
poly Z	5'AAAAAAGGGGGGGGGGGG3'

[0061]

Name	Invention probe
probe w	BODIPY FL/C6-5'AAAAAAAAAGGGGGG3'
probe x	BODIPY FL/C6-5'TTTTTTTTTCCCCCC3'
probe y	BODIPY FL/C6-5'GGGGGGGGGAAAAAA3'
probe z	BODIPY FL/C6-5'CCCCCCCCCTTTTTT3'

[0062]

[Table 3]

Table 3

Nucleic acid probe	Target nucleic acid	Fluorescence intensity from probe alone (A)	Fluorescence intensity after addition of target nucleic acid (B)	Decrease in fluorescence intensity, % (C)*
W	w	330	380	-15
X	x	440	430	2
Y	y	40	50	25
Z	z	360	30	92

* Decrease in fluorescence intensity, % (C) = {(A-B)/A} x 100

As is appreciated from Table 3 and the preceding Example,

[0063]

a substantial decrease takes place in fluorescence intensity (i) when an end of an invention probe labeled with a fluorescent dye is composed of C and hybridization of a target nucleic acid forms a G-C pair, or (ii) when an end of an invention probe labeled with a fluorescent dye is composed of a base other than C and at least one G exists on a side closer to the 3'end of a target nucleic acid than a base pair formed of a base at a location where the invention probe is labeled with the fluorescent dye and a base of the target nucleic acid.

[0064]

Example 4

Concerning the kinds of dyes usable for labeling nucleic acid probes of the present invention, an investigation was made in a similar manner as in the preceding Examples. As an invention probe, the probe z of Example 14 was used. As a target nucleic acid, on the other hand, the oligonucleotide z of Example 14 was employed.

The results are shown in Table 5. As is readily envisaged from this table, illustrative fluorescent dyes suitable for use in the present invention can include FITC, "BODIPY FL", "BODIPY FL/C3", "BODIPY FC/C6", 6-joe, and TMR.

[0065]
[Table 4]

Table 4

Fluorescent dye	Decrease in fluorescence intensity (%)
FITC	90
"BODIPY FL"	95
"BODIPY FL/C3"	98
"BODIPY FL/C6"	97
6-joe	75
TMR	93

Incidentally, the decreases (%) in fluorescence intensity were calculated in a similar manner as in Example 4.

[0066]

Example 5

Preparation of fluorescence quenching probes labeled with a fluorescent dye BODIPY FL, Probe Eu47F and Eu1392R

(5-1) Synthesis of the fluorescence quenching probe Eu47F

The fluorescence quenching probe Eu47F, which was composed of a deoxyribooligonucleotide having the base sequence of (5')CITAACACATGCAAGTCG(3')(I: inosine) and labeled on the phosphate group at the 5'end thereof with "BODIPY FL" as described Example 1.

[0067]

(5-2) Synthesis of Eu1392R

A deoxyribooligonucleotide the base sequence of which was

(5')TTGTACACACCGCCCGTCA(3') was synthesized. The fluorescence-quenching probe Eu1392 according to the present invention was synthesized in a way similar to that in the above (5-1).

[0068]

Example 6

(6-1) Cultivation of *Escherichia coli* JM109

Using Medium 53 (composition: casein peptone (tripsin digest of casein), 10 g; yeast extract, 5 g; glucose, 5 g; salt, 5 g; distilled water, 1000 mL), *Escherichia coli* JM109 was cultivated (culture medium 50 mL/250 mL Erlenmeyer flask, 37℃, 12 hours, shaking culture). Cells were collected from the culture (centrifugation under 10,000 rpm for 5 minutes, washed twice with distilled water).

[0069]

(6-2) Preparation of DNA of 16S rRNA

Using "SOGEN Kit" (NIPPON GENE CO., LTD.), whole RNAs were extracted from the cells in accordance with the protocol of the kit.

Using "BcaBEST™ RNA PCR Kit" (Takara Shuzo Co., Ltd.), the extract was subjected with respect to 16s RNA to amplification and reverse transcription reaction (RT-PCR) under known usual conditions in accordance with the protocol of the kit. Upon these amplification and reverse transcription reaction (RT-PCR), the above-described fluorescence quenching probe EU1392R according to the present invention was used as a primer. Subsequently, RNA was cleaved by Rnase H (30℃, 20 minutes), and pure cDNA of the 16S rRNA gene was obtained. The concentration of cDNA was determined using "OliGreen^{R}ssDNA Quantitation Kit" (Molecular

Probes, Inc.).

[0070]

Example 7

(7-1) Quantitative PCR, data analysis, and preparation of working curves for cDNA

With respect to the above-described cDNA solution, a real-time monitoring quantitative PCR reaction was conducted using the invention fluorescence quenching probe EU47F as a forward primer and the invention fluorescence quenching probe Eu1392R as a reverse primer.

Using "LightCycler™ System" (Roche Diagnostic GmbH, Germany) as a real-time monitoring quantitative PCR system, a reaction was conducted in accordance with the procedures described in the manual. Incidentally, "TaKaRaTaq™" (Takara Shuzo Co., Ltd.) was used as DNA polymerase.

[0071]

PCR was conducted with the following components:

E. coli cDNA	1.0 μL
(final concentration: 10 to 10 copies)	
Primer solution	4.0 μL
(final concentration: 0.1 μM)	
TaKaRaTaq	10.0 (μL 0.5 units)
"MiliQ" purified water	5.0 μL
Final whole volume	20.0 μL

Incidentally, the experiment was conducted using the cDNA in the copy numbers specified in the footnote of FIG. 1. The final concentration of $MgCl_2$ was 2 mM.

[0072]

The reaction was conducted under the following conditions:

Denaturation Initial: 95℃, 60 seconds

Second and onwards: 96℃, 10 seconds

Annealing 50℃, 5 seconds

DNA extension: 72℃, 60 seconds

Measuring conditions were set as follows:

Exciting light: 488 nm

Measuring fluorescent color: 530 nm

[0073]

Real-time monitoring quantitative PCR was conducted under similar conditions as described above, and the intensities of fluorescence in individual cycles was actually measured. The actually measured values were analyzed in accordance with the data analysis method of the present invention. Specifically, the data were processed through the following steps:

(a) The intensity of fluorescence in each cycle in the reaction system in which the amplified nucleic acid hybridized to the nucleic acid primer labeled with the fluorescent dye [namely, the intensity of fluorescence at the time of the nucleic acid extending reaction (72℃)] was corrected in a correction processing step such that the intensity of fluorescence was divided by the intensity of fluorescence in the reaction system measured at the time of complete dissociation of the nucleic acid hybrid complex (the hybrid complex formed by hybridization of the amplified nucleic acid and the nucleic acid primer [namely, the intensity of fluorescence at the time of

completion of the thermal denaturing reaction of the nucleic acid (96℃)], that is, the actually-measured intensities of fluorescence were corrected in accordance with the following formula (1):

$$f_n = f_{hyb,n}/f_{den,n} \qquad (1)$$

where

f_n: a correction value for the intensity of fluorescence in each cycle,

$f_{hyb,n}$: the intensity of fluorescence at 72℃ in each cycle, and

$f_{den,n}$: the intensity of fluorescence at 96℃ in each cycle.

[0074]

(b) A processing step that the values correction-processed by formula (1) in the respective cycles were introduced into the formula (3) to calculate the rates of quenches (%) in fluorescence between the samples in the respective cycles, namely, a step for performing processing in accordance with the following formula (10):

$$F_n = f_n/f_{25} \qquad (10)$$

where

F_n: a processed value in each cycle,

f_n: a value of each cycle as obtained in accordance with formula (1), and

f: a value of the 25th cycle as obtained in accordance with formula (1).

Formula (10) is similar to formula (3) except for a=25.

[0075]

(c) A step that the processed value of each cycle as obtained in step (b) was subjected to processing in accordance with formula (6) to obtain the logarithm of the rate of a change (decrease or quench, %) in fluorescence intensity, namely, a step for performing processing in accordance with the following formula (11):

$$\log_{10}\{(1-F_n) \times 100\} \qquad (11)$$

where

F_n: value obtained in accordance with formula (10).

Formula (11) is similar to formula (6) except for b=10 and A=100.

[0076]

The above results are shown in FIG. 1.

FIG. 1 is a print-out obtained by plotting the values, which have been calculated in steps (a), (b) and (c), against the corresponding cycles.

[0077]

Next, based on the graph of FIG. 1, processing was performed through the following steps (d) and (e).

(d) A step that data processed in step (c) are compared with 0.2 as a threshold, and the number of cycles the data of which reach the threshold is counted.

(e) A step that a graph is prepared by plotting values, which have been calculated in step (d), along X-axis and the numbers of copies before the initiation of the reaction along Y-axis, that is, a working line (FIG. 2) for *Escherichia coli* cDNA is prepared.

FIG. 2 shows the final results obtained when data obtained by the

quantitative PCR method of the present invention were processed by the data analysis method of the present invention, namely, through steps (a), (b), (c), (d) and (e). It is understood that concerning a nucleic acid sample the number of copies of which is unknown, the number of copies before the initiation of the reaction can be determined with good accuracy from FIG. 2.

[0078]

Example 36

(8-1) Construction of a polymorphous system (a co-cultivation system of microorganisms)

Ten (10) bacteria strains shown in Table 5 were purchased from DSMZ (Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH). Using Medium 53 described above, they were separately cultured. Culture conditions were similar to the above-described conditions for *Escherichia coli*. From each culture, cells were collected (centrifugal separation at 10,000 rpm for 10 minutes; washed twice with distilled water). From each sample of cells, whole RNAs were extracted in a similar manner as described above by using "SOGEN Kit" (NIPPON GENE CO., LTD.).

[0079]

Table 5

Strain No.	DSMZ No.	HhaI fragment (bp)	Molar fraction (%) determined from T-RFLP	Number of quantitated copies	Number of quantitated copies/number of initially added copies
1	65	22	9.5	27400	0.91
2	3138T	43	10.9	31400	1.05
3	12778	52	9.7	27900	0.93
4	20530	104	9.4	27100	0.90
5	50108	168	10.4	30000	1.00
6	20152	332	9.3	26800	0.89
7	43879	404	9.7	27900	0.93
8	20579	432	9.9	28500	0.95
9	5078	531	10.4	30000	1.00
10	43673	626	10.8	31100	1.04

[0080]

1: Paracoccus pantotrophus

2: Sphingomonas natatoria

3: Bdellovibrio stolpii

4: Microbacterium imperiale

5: Pseudomonas fluorescens

6: Agromyces medislanum

7: Cellulomonas cellulans

8: Brevibacterium liquefaciens

9: Leminorella grimontii

10: Rhodococcus luteus

[0081]

In a similar manner as in the above-described case of *Escherichia coli*, pure cDNAs of the 16S rRNA genes of the respective strains were obtained. The respective concentrations of the thus-obtained cDNAs of the 10 strains were determined in a similar manner as in the above-described case of *Escherichia coli*. The solutions the cDNA concentrations of which had been ascertained were diluted with distilled water to 300,000 copies/μL. Concerning the 10 strains, the diluted solutions were mixed in equal amounts to provide a co-cultivation system of microorganisms, in other words, a polymorphous system (hereinafter called a "polymorphous system"). As the cDNAs of the 10 strains are each contained at the concentration of 300,000 copies/μL in the polymorphous system, the cDNAs are contained as a whole at a concentration of 3,000,000 copies/μL.

[0082]

(8-2) Real-time monitoring quantitative PCR

With respect to the cDNAs in the above-described polymorphous system, real-time monitoring quantitative PCR was conducted in a similar manner as in the above-described *Escherichia coli* by using the fluorescent quenching probes Eu47F and Eu1392R of the present invention as primers common to the strains.

A polymorphous sample was added to a reaction mixture to give 300,000 copies/20 μL in terms of absolute count. In real-time monitoring quantitative PCR of the polymorphous system, the reaction was terminated in the 22nd cycle in which a decrease in the intensity of fluorescence was observed and which was an exponential growth phase of the genes (see FIG. 30). The number of copies of cDNAs in the reaction mixture of real-time monitoring quantitative PCR conducted on the polymorphous system was 288,000 copies when he threshold was set as log R_n (fluorescence quenching rate) = 0.2 (see FIG. 31). Since the initially-added amount, that is, the calculated count was 300,000 copies, the working line prepared by the method of the present invention has been confirmed to show good quantitativeness.

[0083]

Example 9

Polymorphous analysis

(9-1) Analysis by T-RFLP

After a PCR reaction was conducted as described above, amplified products were purified using a column ("Microcon PCR"; Millipore Corporation, Bedford, MA, U.S.A.). Purified products were treated

overnight with a restriction endonuclease HhaI (recognition site: GCG/C, /: cleaved site). After completion of the treatment, only cleaved fragments were purified through columns ("Microcon" and "Micropure-Ez"; Millipore Corporation, Bedford, MA, U.S.A.). The sizes of cDNA fragments of the respective strains after the treatment with the restriction endonuclease are shown in Table 5.

The cDNA solution, to which the column chromatographic purification had been applied, was subjected to thermal denaturation, followed by a T-RFLP analysis by a sequencer ("ABI PRISMTH 310"; Perkin Elmer – Applied Biosystems Inc.). A peak pattern of the T-RFLP analysis is shown in FIG. 3. Each peak was quantitated using a standard "BODIPY FL"-modified fragment the concentration of which was known. The molar fractions (%) of the individual peaks were determined. As a result, the molar fractions (%) all fell within a range of from 9.4 to 10.8 and no substantial difference was observed in the efficiencies of PCR amplification of the cDNA fragments of the respective strains (see Table 5). The ratio of the number of quantitated copies to the number of initially added copies ranged from 0.89 to 1.04 (see Table 5). It has hence been found that initial copies of polymorphous strains in a polymorphous system can be accurately quantitated by this method.

[0084]

[Advantageous Effect of Invention]

The quantitative, polymorphous analysis method according to the present invention has the above constitution; and the method has dvantageous effects as will be set out below.

1) The quantitative, polymorphous analysis method can easily and quickly perform with good quantitativeness the determination of the amount of a target gene or the polymorphous composition of the gene.

2) The quantitative PCR method according to the present invention is a novel method and is excellent in quantitativeness.

3) The method for analyzing the data obtained by the quantitative PCR method also is a novel method and can correctly determine the number of initial copies of a gene before its PCR amplification.

4) In the novel quantitative PCR method according to the present invention, the amplified nucleic acid is labeled with a fluorescent dye: in the polymorphous analysis, the fluorescent dye can be utilized as a marker.

5) As results of the above 2) to 4), the quantitative, polymorphous analysis method becomes to be a polymorphous analysis method, which is particularly excellent in quantitativeness.

[Brief Description of Drawings]

[Fig. 1] FIG. 1 shows amplification curves of 16S rRNA genes (cDNAs) obtained using quantitative PCT according to the present invention;

[Fig. 2] FIG. 2 illustrates a working line for cDNA, which was prepared by a data analysis method according to the present invention

[Fig. 3] FIG. 3 illustrates an analysis pattern by polymorphous T-RELP according to the present invention;

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[Brief Description of Drawings]

[Fig. 1] FIG. 1 shows amplification curves of 16S rRNA genes (cDNAs) obtained using quantitative PCT according to the present invention.

Solid line: cDNA of Escherichia coli; dotted line: cDNA of polymorphous system. 10^2, 10^3, 10^4, 10^5, 10^6: the copy number

[Fig. 2] FIG. 2 illustrates a working line for cDNA, which was prepared by a data analysis method according to the present invention

a: 288000 copies

[Fig. 3] FIG. 3 illustrates an analysis pattern by polymorphous T-RELP according to the present invention;

bp: the number of base pair

[Name of Document] ABSTRACT

[Abstract]

[Problem to be Solved] To provide a polymorphous analysis method with excellent quantitativeness.

[Means to Solve] There is provided with a polymorphous analysis method, in which the method comprises: amplifying a target nucleic acid by using a novel quantitative PCR method using a fluorescence-quenching probe; and performing a polymorphous analysis in respect to the amplified product. It is to possible to determine a starting copy number of a gene before the amplification by using the data analysis method according to the present invention. That is, the determination is performed by a method for analyzing data obtained by the quantitative PCR method, comprising a processing step for correcting a fluorescent intensity value on nucleic acid extending reaction with a fluorescent intensity value on completed heat-denatured reaction.

[Selected Drawing] None.